

**Amendments to the Specification:**

Please replace the paragraph beginning the last 4 lines of page 52 through page 53, line 7 with the following replacement paragraph:

Although the Aga2p-HA fusion is simply a construction intermediate towards the final Aga2p-HA-antibody fusion, it does provide a means for confirming that a fusion peptide is anchored and accessible on the cell surface in this system. Anchorage of the HA peptide to the external cell wall by the fusion has been verified by immunofluorescent staining of whole unfixed cells with the 12CA5 mAb (Boehringer Mannheim, Indianapolis, IN), detected by flow cytometry and fluorescence microscopy (Figure 9). Since whole 12CA5 antibody molecules bind to the HA epitope without any disruptive biochemical treatment of the cell wall, the Aga2p is accessible to the cell exterior for macromolecular recognition.

Please replace the paragraph on page 53, line 8, through page 53, line 15 with the following replacement paragraph:

As described previously, the Aga2p binding subunit is attached to the cell wall through disulfide bonds to Aga1p, which is covalently anchored to other cell wall components. Treatment with DTT abolishes labeling with 12CA5, indicating that the Aga2p-HA fusion is attached to the cell surface by disulfide bonds. The AGA1 gene was cloned by PCR and subcloned downstream of the GAL1 promoter. Expression of AGA1 was induced by switching to galactose growth media.

Please replace the paragraph on page 53, line 16, through page 54, line 4 with the following replacement paragraph:

The HA epitope tag was included in antibody fusions, to enable double fluorescence labeling for both surface antibody levels and binding of fluorescently-labeled antigens. This approach decouples cell-to-cell variations in antibody expression level from single-cell measurements of antigen affinity. For example, indirect immunofluorescence with phycoerythrin-labeled secondary IgG against the  $\alpha$ -HA monoclonal antibody provides a measure of surface antibody numbers, while fluorescein-labeled antigen bound to the antibodies provides a measure of binding affinity. Because  $\approx 10^4$  copies of a-agglutinin are displayed per cell, stochastic effects on binding measurements are minimal. Since commercial flow cytometers can detect under  $10^3$  fluorophore molecules, signal-to-noise ratio should not be problematic. The ratio of green fluorescence (fluorescein, i.e., antigen binding) to red fluorescence (phycoerythrin, i.e. antibody number) is proportional to the fraction of antibodies bound by antigen.

Please replace the paragraph on page 55, last 5 lines, through page 56, line 8 with the following replacement paragraph:

As a model system for development of the yeast surface display library screening method, we have displayed a functional anti-fluorescein scFv and *c-myc* epitope tag on the cell wall of yeast by fusion to  $\alpha$ -agglutinin, which unlike  $\alpha$ -agglutinin is a two-subunit glycoprotein (Fig. 2). The 725 residue Aga1p subunit anchors the assembly to the cell wall (Roy, 1991) via  $\beta$ -glucan covalent linkage (Lu, 1995); the 69 amino acid binding subunit Aga2p is linked to Aga1p by two disulfide bonds (Cappellaro, 1994). The native  $\alpha$ -agglutinin binding activity is localized to the c-terminus of Aga2p (Cappellaro, 1994); thus, this represents a molecular domain with accessibility to extracellular macromolecules and a useful site for tethering proteins for display. A vector for displaying proteins as C-terminal fusions to Aga2p was constructed (Fig. 3).

Please replace the paragraph on page 56, beginning with "Example 21", through page 57, line 2 with the following replacement paragraph:

#### **EXAMPLE 21**

##### **Verification of expression and surface localization of scFv**

Expression of the Aga2p-scFv fusion is directed by the inducible GAL1 promoter (Johnston, 1984). Growth of yeast on glucose medium allows essentially complete repression of transcription from the GAL1 promoter, an important consideration for avoiding counterselection against sequences which negatively influence host growth. Switching cells to medium containing galactose induces production of the Aga1p and Aga2p fusion gene products, which associate within the secretory pathway and are exported to the cell surface. Surface localization of the Aga2p-scFv fusion has been verified by confocal fluorescence microscopy and flow cytometry. Cells labeled simultaneously with an anti-*c-myc* mAb and fluorescein-conjugated dextran (FITC-dextran) were examined by laser scanning confocal microscopy (Fig. 8). Control cells bearing a vector which directs display of an irrelevant peptide (i.e., a hemagglutinin (HA) epitope tag only) are not labeled by mAb specific for the *c-myc* epitope or FITC-dextran (Fig. 8A).

Please replace the paragraph on page 64, beginning with "Example 25", through page 65, line 11 with the following replacement paragraph:

#### **EXAMPLE 25**

##### **Induction and detection of scFv-KJ16 on the yeast surface**

Yeast cells transformed with pCT202/scFv-KJ16 were grown overnight at 30°C with shaking in 3 ml selective glucose medium SD-CAA (glucose 2 wt %, Difco yeast nitrogen base 0.67 wt %, casamino acids 0.5 wt %). After ~18-20 hours, recombinant AGA1 + AGA2-scFv expression was induced at 20°C with shaking in 5 ml selective galactose medium (SG-CAA, where 2% galactose replaces the glucose in SD-CAA).

Cultures were harvested after ~20-24 hours (1-2 doublings) by centrifugation, washed with PBS (10 mM NaPO<sub>4</sub>, 150 mM NaCl, pH 7.3) containing 0.1% bovine serum albumin and 0.05% azide, and incubated 45 minutes on ice with 25 mL of 10 mg/ml anti-HA Mab 12CA5 (Boehringer Mannheim, Indianapolis, IN), anti-c-myc Mab 9E10 (1:100 dilution of raw ascites fluid; Berkeley Antibody Co., Richmond, CA), or biotinylated-scTCR [~360 nM] prepared from inclusion bodies expressed in *E. coli* (Schodin et al., 1996). Cells were washed with PBS and incubated 30 minutes on ice with either FITC-labeled F(ab')<sub>2</sub> goat anti-mouse IgG (1:50; Kirkegaard and Perry Labs, Inc., Gaithersburg, MD) or a streptavidin-phycoerythrin (SA-PE) conjugate (1:100; PharMingen, San Diego, CA). Labeled yeast cells were analyzed on a Coulter Epics XL flow cytometer at the Flow Cytometry Center of the UIUC Biotechnology Center. Event rate was ~250 cells/sec. Data for 10,000 events was collected, and the population was gated according to light scatter (size) to prevent analysis of cell clumps. These conditions were also used to generate equilibrium antigen binding isotherms after incubation of scFv-KJ16 yeast with various dilutions of scTCR. Scatchard analysis was performed to determine the K<sub>D</sub> values, using the estimated concentration of the biotinylated-scTCR and mean fluorescence units taken directly from flow data.

Please replace the paragraph on page 65, beginning with "Example 26", through page 66, line 13 with the following replacement paragraph:

#### **EXAMPLE 26**

##### **Production of a scFv-KJ16 random mutant library**

Approximately 50 ng of pCT202/scFv-KJ16 were transformed into *E. coli* XL1-Red cells (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Following a 1 hour induction in SOC medium, the recovery was centrifuged at 2000 rpm for 5 minutes and resuspended in 500 ml of liquid LB medium containing 100 mg/ml ampicillin plus 50 mg/ml carbenicillin (LB-AMP100-CARB50). The resuspension was added to 15-ml LB-AMP100-CARB50 in a 50-ml Erlenmeyer flask and grown at 37°C with shaking. The culture was replenished with a fresh 15-ml LB-AMP100-CARB50 at mid-log phase (OD<sub>600</sub> ~ 0.2-0.4), then grown to saturation (OD<sub>600</sub> ~ 1.0-1.1; this was considered one "cycle" or round of mutation). A small fraction of this culture (0.75 ml) was added to the next cycle (15-ml LB-AMP100-CARB50). After six cycles of growth, Wizard Miniprep (Promega, Madison, WI) DNA plasmid preparations were performed on the 15-ml culture. Approximately 4.5 mg of pCT202/scFv-KJ16 DNA from cycle six were transformed into each of 3 tubes of yeast strain EBY100 using the LiAc method (Gietz et al., 1995). The 3 reactions were pooled and after resuspension in 1-ml ddH<sub>2</sub>O, 1/2000 of the pool plated on selective plates to determine transformation efficiency. Fifty milliliters of SD-CAA were inoculated with the remainder of the culture, grown overnight at 30°C with shaking, passaged to OD<sub>600</sub> = 0.05, and grown overnight at 30°C to OD<sub>600</sub> > 1.0. Five milliliters of SG-CAA were then inoculated to OD<sub>600</sub> ~ 0.5 and grown overnight at 30°C with shaking to OD<sub>600</sub> = 1.0-2.0.

Please replace the paragraph on page 66, beginning with "Example 27", through page 67, line 4 with the following replacement paragraph:

#### **EXAMPLE 27**

##### **Selection of scFv-KJ16 mutant library by FACS**

Cells were double-labeled as described above with anti-c-*myc* Mab and biotinylated-scTCR (used at a concentration ~10 nM). The reaction volume was adjusted to maintain ~10-fold molar excess of antigen (scTCR) over surface scFv. Samples were sorted on a Coulter 753 bench with a sort window as shown in Figure 3 and an event rate of 4,000 cells/sec. A total of  $8 \times 10^7$  cells were examined during the first sorting round, with 0.1-0.4% of the population collected. The collected cells were regrown at 30°C in SD-CAA and switched to SG-CAA prior to the next round of sorting. A total of 4 rounds of sorting was performed, with the first 2 sorts in enrichment mode (high recovery of all positive clones) and the last 2 sorts in purification mode (coincident negative cells rejected). Immediately following the last sort, the collected cells were re-sorted and plated on selective plates to isolate individual clones.

Please replace the paragraph on page 68, lines 1-11 with the following replacement paragraph:

The monoclonal anti-TCR antibody KJ16 recognizes a conformational epitope on the Vb8 chain of the TCR (Brodnicki et al., 1996). KJ16 has been used for many *in vivo* studies in mice, including efforts to target and delete the Vb8 population of T cells (Born et al., 1987, McDuffie et al., 1986, Roehm et al., 1985). To evaluate the possible effects of varying antibody affinity in mediating these effects, the use of a yeast display system to identify KJ16 variants with increased affinity for TCR was examined. The scFv gene from the anti-TCR antibody KJ16 has been cloned previously and the scFv protein exhibited approximately the same affinity,  $K_D \sim 120$  nM, as KJ16 Fab fragments (Cho et al., 1996).

Please replace the paragraph on page 68, lines 12-27 with the following replacement paragraph:

The scFv-KJ16 coding sequence was subcloned so as to be expressed as a fusion polypeptide with the Aga2p agglutinin subunit expressed on the yeast cell surface. The fusion polypeptide includes a hemagglutinin (HA) epitope tag N terminal to the scFv and a c-*myc* epitope tag at the carboxy-terminus. The inclusion of these epitopes allows monoclonal anti-HA (12CA5) and anti-c-*myc* (9E10) antibodies to be used in flow cytometry to quantify surface expression of the full length scFv independently of antigen-binding activity. Such normalization helps account for the effects of cell-to-cell variability in surface levels of the fusion polypeptide. As discussed below, the availability of two independent epitope tags can also control for the selection of

individual epitope mutants that might not be desired in screening for ligand binding mutants. To evaluate the binding properties of cell surface scFv, a soluble single-chain Vb8-Va3 TCR (Schodin et al., 1996) was biotinylated and the bound ligand was detected with a phycoerythrin-streptavidin conjugate.

Please replace the first paragraph on page 69 with the following replacement paragraph:

Figure 12 shows that yeast transformed with the scFv-KJ16/Aga2 plasmid expressed the HA epitope (Figure 12A) and the *c-myc* epitope (Figure 12B). Control yeast transfected with only the Aga2p/HA expression vector were positive for the anti-HA Mab but not for the anti-*c-myc* antibody. The fraction of cells in the non-fluorescent population has been found to depend on plasmid stability and culture growth phase (data not shown), but the physiological processes that are involved are unknown. Nevertheless, decreasing the induction temperature to 20°C and decreasing the induction time to less than two culture doublings produces populations with >75% of the cells displaying the scFv-KJ16. scFv-4-4-20 was displayed with this system with approximately the same proportion of positive cells.

Please replace the second paragraph on page 69 with the following replacement paragraph:

Binding of biotinylated scTCR to cell surface scFv was also detected by flow cytometry (Figure 12C). The fraction of cells that expressed active scFv was similar to that detected with anti-HA and *c-myc* antibodies, consistent with the expression of full length, properly folded scFv. Furthermore, two-color histograms demonstrated a tight correlation of scTCR binding with both HA and *c-myc* epitope display (data not shown). Biotinylated-scTCR binding is specific to yeast displaying the scFv-KJ16, and was completely inhibited by excess soluble KJ16 IgG (Figure 12D).

Please replace the paragraph on page 69, line 22 through page 70, line 6 with the following replacement paragraph:

The approximate affinity of the surface displayed scFv-KJ16 was determined *in situ* on the cell wall by titrating whole cells with varying concentrations of biotinylated scTCR. Equilibrium binding was measured by analyzing cell-bound scTCR by flow cytometry. Scatchard analysis of the binding data (Figure 13) yielded a  $K_D$  of 500 nM, within five fold of that observed for soluble scFv-KJ16. Such agreement is reasonable, since  $K_D$  was calculated under the assumption that 100% of the scTCR was active, likely to be an overestimate (i.e., if only 20% were correctly folded, then the surface scFv would have a  $K_D \sim 100$  nM). Previously, a substantial fraction of the scTCR purified from solubilized *E. coli* inclusion bodies is incorrectly folded was found (Schodin et al., 1996).

Please replace the paragraph on page 69, beginning with "Example 30", with the following replacement paragraph:

### **EXAMPLE 30**

#### **Selection of Mutagenized scFv-KJ16/Yeast by Fluorescence-Activated Cell Sorting**

An *E. coli* mutator strain has been used to mutagenize an scFv for affinity maturation by phage display (Low et al., 1996). This approach was successful in identifying a mutant of scFv-4-4-20 with higher affinity for fluorescein using yeast display. A strength of this mutagenesis approach is its simplicity, requiring only *E. coli* transformation and cell growth. Furthermore, the *E. coli* mutator strain introduces mutations throughout the expression plasmid, and therefore does not bias changes to portions of the scFv believed to be important for determining binding characteristics. Whether this aspect of mutator strain mutagenesis is advantageous depends on the ability to identify key residues that might influence antigen binding, based on available structural information. Examination of published affinity maturation studies suggest that the location of such residues, generally in non-contact residues, is not yet predictable *a priori* (Hawkins et al., 1993, Patten et al., 1996, Schier et al., 1996, Thompson et al., 1996, Yang et al., 1995, Yelton et al., 1995).

Please replace the first paragraph on page 71 with the following replacement paragraph:

To apply this strategy to scFv-KJ16, the scFv-KJ16/Aga2 plasmid was propagated in the *E. coli* mutator strain XL1-Red (Stratagene) for six cycles of growth. This procedure was predicted to introduce an average of two to three point mutations in the scFv coding sequence, based on a mutation rate per cycle of 1 in 2000 bps. The resultant plasmid preparation was transformed into yeast yielding a library size of approximately  $3 \times 10^5$  transformants. In other work, larger libraries ( $10^7$ ) have been obtained by further optimization of transformation procedures and by pooling independent transformations. This number does not represent an upper size limit for library construction, as further efforts at optimization and scaleup could be straightforwardly applied.

Please replace the paragraph on page 71, line 13 through page 72, line 9 with the following replacement paragraph:

The mutagenized yeast library was subjected to four successive cycles of sorting and amplification, using a double stain for anti-c-myc antibody binding (FITC) and biotinylated-scTCR binding (PE). Biotinylated TCR was used at a 1:5000 dilution (~10 nM) that yielded just below the detectable threshold of binding by wt scFv-KJ16/yeast (Figure 13). The two channel fluorescence profiles of the mutated scFv-KJ16 sample after one sorting cycle (Figure 14A) and after four sorting cycles (Figure 14B) are shown. Cells that exhibited fluorescence above the diagonal window shown in Figure

14 were collected for regrowth. The rationale for this diagonal window was that in any given round the sort criteria were based on antigen binding per displayed polypeptide fusion. for example, selection based only on higher PE fluorescence levels (i.e., scTCR binding) would include not only those mutants with higher affinity scFv, but those that display a higher density of scFv per yeast cell. The latter mutants would in principle be eliminated by including the anti-c-*myc* antibody as one of the two parameters to normalize for surface expression variability. The first two sorting rounds were performed in enrichment mode, isolating the ~0.5% of the cell population with the highest fluorescence and not setting the sort software to reject coincidences (two cells in the same sorted droplet). The final two sorting rounds were performed for purity, with high coincidence rejection. After the fourth cycle, cells were resorted immediately and plated. Ten colonies (mut1 – 10) were selected for further analysis.

Please replace the paragraph on page 72, beginning with “Example 31”, through page 73, line 4 with the following replacement paragraph:

### **EXAMPLE 31**

#### **Characterization of Mutant scFv-Yeast**

Each of the 10 selected mutants were labeled with anti-HA antibody, anti-c-*myc* antibody, and biotinylated-TCR and was analyzed by flow cytometry (Figure 15). As might be expected, one clone (mut6) appeared phenotypically similar to wt scFv-KJ16/yeast. Another clone (mut7) was found to exhibit higher TCR binding levels, a result confirmed by several independent titrations. Finally, a number of the mutants (mut1-5, 8, 9) consistently showed reduced binding to the anti-c-*myc* antibody compared to binding of the anti-HA antibody or the biotinylated scTCR. The presence of this class of mutants could be explained by the diagonal sort window specification: as shown in Figure 14, cells can “move” into the sort window either by increasing scTCR (PE) binding at constant c-*myc* (FITC) signal, or alternatively by decreasing c-*myc* (FITC) binding at constant scTCR (PE) signal. The selection of these mutants could be easily circumvented by using both epitope tags in the fusion, HA and c-*myc*. Thus, by alternating labeling of each of these epitope tags in each round of sorting, diminished binding to one of the epitope tags would not be enriched in consecutive sorting rounds as in this case.

Please replace the paragraph on page 73, lines 5-21, with the following replacement paragraph:

Fluorescence histograms of the presumptive c-*myc* epitope mutant (mut4), the scTCR binding mutant (mut7) and another mutant (mut10) were compared with the wt scFv (Figure 16). Mut4 (Figures 16A and 16B) showed a reduction in anti-c-*myc* labeling, mut7 showed enhanced scTCR binding (Figures 16C and 16D), and mut10 did not show a shift in either, but the fraction of cells that were positive was higher than with the

wt scFV (Figures 16E and 16F). As shown in Figures 16E and 16F, close to 100% of mut10 cells were positive for each of the agents tested. This contrasts with each of the other mutants (e.g., see mut4 and mut7) which resembled the wt scFv-KJ16 yeast in exhibiting two distinct populations of cells, one with reduced levels of cell surface scFv. Enhanced plasmid stability of mut10 and repeated failures to rescue the expression plasmid from mut10 into *E. coli* suggest that chromosomal integration has occurred with this mutant plasmid. Thus, the altered surface expression characteristics of mut10 appear to be a consequence of integration of the expression plasmid.

Please replace the paragraph on page 73, line 22, through page 74, line 2 with the following replacement paragraph:

Binding affinity to scTCR was estimated for the mutants shown in Figure 16 by titration with soluble biotinylated scTCR (Figure 17). Nonlinear curve fitting of this data indicate unaltered  $K_D$  for mut4 and mut10, but a threefold increased affinity for mut7. The increase in mean fluorescence of mut10 is due to the absence of a nonfluorescent tail in the distribution rather than increased scTCR binding, as is evident in Figures 16E and 16F.

Please replace the paragraph on page 74, beginning with "Example 32", with the following replacement paragraph:

### **EXAMPLE 32**

#### **Sequences of Mutant scFV**

The nucleotide sequences of the wt-scFv-KJ16 cloned into the yeast display plasmid, and mut4 and mut7 following rescue of the plasmids from yeast was determined (Figure 18). The wt scFv-KJ16 contained two silent changes from the originally published scFv Sequence (Cho et al., 1995). These may have been introduced by PCR prior to cloning of the scFv into the yeast display plasmid. The mut4 sequence contained one mutation and mut7 contained two mutations. The only mutation in mut4 was present in the *c-myc* epitope (Lys to Glu), consistent with its reduced binding by anti-*c-myc* antibody as described above. Mut7 contained a change from Arg to Lys in a framework region of the  $V_L$  region and a change from Ser to Arg in CDR1 of the  $V_L$  chain. The latter mutation is consistent with the higher binding affinity observed for mut7.

Please replace the paragraph on page 74, beginning with Discussion, through page 75, line 14 with the following replacement paragraph:

#### **Discussion**

Phage display has been used for the selection of scFv with higher antigen binding affinity, as well as isolation of new scFv's from naive libraries (Hoogenboom, 1997).



However, there have been difficulties in the expression of some mammalian proteins in *E. coli*, in part because of toxicity, codon bias, or folding problems (e.g. Knappik & Pluckthun, 1995, Ulrich et al., 1995, Walker & Gilbert, 1994). Yeast expression can potentially obviate some of these problems, by offering the advantage that proteins can be expressed with eucaryotic post-translational modifications (e.g., glycosylation and efficient disulfide isomerization). Furthermore, phage display does not generally possess the quantitative precision to discriminate between mutants with binding affinity differing by less than five-fold (Kretzschmar et al., 1995). By contrast, fluorescence labeling and sorting allowed the isolation of 4-4-20 scFv clones with only 3 fold increased affinity. Since most large changes in antigen binding affinity result from directed combination of point mutations, each with smaller effects (Hawkins et al., 1993, Schier et al., 1996, Yang et al., 1995), the capability to identify subtle improvements in affinity could be of significant value. With these advantages in mind, the use of a yeast display system for the affinity maturation of an anti-T cell receptor scFv was developed.

Please replace the paragraph on page 75, line 15, through page 76, line 6 with the following replacement paragraph:

A scFv that is specific for the Vb8 region of a mouse TCR was used in order to generate anti-TCR reagents that may ultimately have enhanced T cell targeting properties *in vivo* (Cho et al., 1997, Cho et al., 1995). The active scFv was expressed as an Aga2p fusion protein on the surface of the yeast, with an affinity that was similar to the native scFv (~500 nM compared to 120 nM for the scFv). To select higher affinity scFv, random mutagenesis with a DNA-repair deficient strain of *E. coli* yielded a mutation frequency of ~2 to 3 per 1000 base pairs after six growth cycles. Flow cytometry with fluorescently labeled scTCR and anti-c-myc antibodies was used to sort cells displaying scFv's with increased scTCR affinity. The anti-c-myc antibody was included as a second criteria for selection to control for mutants with increased TCR binding due not to higher affinity but because of higher cell surface expression of the scFv-c-myc fusion. After multiple rounds of selection, three mutant phenotypic classes were observed: 1) reduced binding to the c-myc antibody but unaltered scTCR binding (mut 1-5, 8, 9); 2) enhanced binding to the scTCR with unaltered c-myc labeling (mut7); and 3) higher efficiency surface expression due to chromosomal vector integration (mut10).

Please replace the paragraph on page 76, lines 7-17 with the following replacement paragraph:

The isolation of classes of mutants that are represented by mut4 and mut7 could be predicted from the selection criteria illustrated in Figure 14. That is, any mutant cell that was identified above the diagonal sort window boundary could be accounted for by either of the properties described for mut4 and mut7, since either an increase in scTCR (PE) signal or a decrease in c-myc (FITC) signal places a cell in the sorting window. This does not represent a substantial problem for this approach, however, because of the availability of two independent epitope tags. By utilizing the HA and c-myc tags in

alternating sorting cycles, progressive enrichment for diminished labeling of one of the epitope tags should not occur.

Please replace the paragraph on page 76, line 18, through page 77, line 5 with the following replacement paragraph:

The isolation of epitope tag mutants highlights an additional application for yeast surface display: mapping of epitopes recognized by monoclonal antibodies. Although alternative strategies that use peptide libraries have been successful in this regard for linear epitopes (Daniels & Lane, 1996), the approach described here can be extended to conformational epitopes. Accordingly, a properly folded protein can be displayed on the yeast cell surface and straightforward random mutagenesis as described herein can be applied to identify epitope residues from non-contiguous polypeptide sequence. Since nonfolded proteins are retained and degraded by the eucaryotic secretory quality control apparatus and varied expression levels are identified by HA or *c-myc* labeling, false identification of epitope residues should be minimized by this procedure. The described approach is substantially easier than alanine scanning mutagenesis.

Please replace the paragraph on page 77, lines 6-13 with the following replacement paragraph:

It is not clear why mut10 was enriched in this screen, since its average single chain T cell receptor labeling per *c-myc* labeling was unaltered. It is possible that the higher fraction of positively labeled cells biased this clone for enrichment due to random spillover into the sort window. In any case, neither scTCR or *c-myc* labeling were different for this clone, and structural rearrangements of the expression plasmid indicate that it had integrated into a chromosome.

Please replace the paragraph on page 77, lines 14-25 with the following replacement paragraph:

The identification of a single unique CDR mutation in mut7 is consistent with the finding that this mutant scFv has enhanced binding to the T cell receptor. Future efforts to obtain only scFv with higher affinity for the T cell receptor (and not *c-myc* mutants) involves alternate selection with anti-HA and anti-*c-myc* antibodies to control for cell surface levels of the scFv. This strategy, combined with DNA shuffling techniques among selected mutants (Stemmer, 1994), should allow the isolation of scFv-KJ16 with considerably higher affinity than the wt scFv ( $K_D \sim 120$  nM). Such mutant KJ16 scFv's can be used to test T cell signaling kinetic phenomena, as well as targeting of T cell-mediated killing via bi-specific antibodies (Cho et al., 1997, Rabinowitz et al., 1996).

Please replace the paragraph on page 77, line 26, through page 78, line 19 with the following replacement paragraph:

The present invention demonstrates the purposeful isolation of affinity matured antibodies via cell surface display. As described above, off-rate selection was employed to identify mutants with decreased dissociation rates, whereas in the expression of scFv-KJ16, equilibrated antigen binding was used. These two approaches are complementary, and depend on the affinity of the starting scFv. For  $K_D > 1$  nM, it is reasonable to pursue the strategy of equilibration with soluble labeled antigen as dissociation rates would be too rapid to allow effective discrimination of kinetic variation. Furthermore, at these lower affinities bulk soluble antigen is not substantially depleted from the labeling reaction mix, given that displayed scFv is present at effective concentrations of approximately 1-10 nM. By contrast, tightly binding antibodies such as 4-4-20 ( $K_D = 0.4$  nM) would deplete soluble labeled antigens at concentrations below  $K_D$  unless inconveniently large labeling volumes were employed. However, dissociation kinetics for such tightly binding antibodies are slow enough to enable quenching, sorting, and analysis via manual mixing procedures. Thus, one could employ a strategy whereby scFv's would be affinity matured via cycles of equilibrium-based screening and mutagenesis to reach  $K_D \sim 1$  nM, followed by cycles of off-rate screening and mutagenesis to obtain still further improvement.

Please replace the paragraph on page 78, line 20, through page 79, line 3 with the following replacement paragraph:

Cell surface display and flow cytometric screening allows selection of clones from a library based on kinetic binding parameters such as  $K_D$  and the dissociation rate constant ( $k_{diss}$ ). Binding parameters of selected mutants may then be quantitatively estimated *in situ* in the display format without a need for subcloning or soluble expression, as shown in Figure 17. By contrast, selection of phage displayed antibodies often involves increasingly stringent wash and elution conditions, even to the extent of pH 2 and 8 M GuHCl. Such stringency selection has poor quantitative precision and may not always relate directly to binding parameters such as  $K_D$  or  $k_{diss}$  under ambient or physiological conditions.

Please replace the paragraph on page 79, lines 4-16 with the following replacement paragraph:

Bacterial cell surface display systems have been described (Gunneriusson et al., 1996) for engineering of antibodies and other proteins. These systems possess some of the advantages of the present yeast display system, although they do not provide the post-translational processing capabilities of the eucaryotic secretory pathway. Access of macromolecules to the displayed protein on bacteria may also be restricted by the diffusion barrier presented by the lipopolysaccharide layer (Roberts, 1996). For this reason, binding to soluble protein antigens or epitope tag labeling with monoclonal antibodies is not possible. Surface display systems in cultured mammalian cells are also available (Rode et al., 1996), but construction and screening of combinatorial libraries for these systems are not as rapid or as versatile as for yeast.

Please replace the paragraph on page 79, line 17, through page 80, line 4 with the following replacement paragraph:

A fairly small library ( $3 \times 10^5$ ) was screened to isolate the mutants described herein. This does not represent an upper limit on yeast library size. Yeast libraries with  $10^7$  clones have been constructed and further increases in library size, if necessary, would be attainable. The present invention shows that yeast surface display can be used to isolate a mutant scFv with increased affinity and that mutants with altered mAb epitopes can be enriched or excluded as desired. Further, the  $K_D$  can be estimated *in situ* in the display format without necessitating subcloning and soluble expression. Quantitative optimization of the screening conditions will enable further improvements in this method. Applications of yeast surface display extend beyond antibody affinity maturation, to the isolation of binding domains from cDNA expression libraries, or isolation of mutant receptors or ligands on the direct basis of kinetic and equilibrium binding parameters.

Please replace the last paragraph on page 80, line 17, through page 81, line 7 with the following replacement paragraph:

Protein engineering has not reached a level of development that allows rational and directed engineering of increased affinity binding. As a result, approaches have been developed that identify improved mutants from large mutant populations. The most widely used approach is "phage display," which has been used to engineer antibodies, especially in the form of linked, "single-chain" antibodies. However phage display methodology has been unable to display single-chain T cell receptors (scTCRs) successfully. This is most likely because folding of isolated single-chain T cell receptors is very inefficient in the absence of the other components of the CD3 complex and the protein folding machinery of the eucaryotic endoplasmic reticulum; the bacterial periplasm is unable to effectively fold these fragments.

Please replace the paragraph on page 81, lines 8-18 with the following replacement paragraph:

The establishment of a yeast surface displayed T cell receptor is illustrated in Figures 19 through 21. A key improvement has been to isolate a mutant T cell receptor which can be displayed in this system. The wild-type T cell receptor is not functionally displayed as shown by the absence of binding by an antibody (1B2) that is specific for the native conformation of the T cell receptor (Figure 19). By mutating the T cell receptor and screening a library for 1B2 binding, a mutant single chain T cell receptor displayed in yeast was identified. This establishes a system which can now be used to isolate mutant single chain T cell receptors with improved binding properties.

Please replace the paragraph on page 81, lines 19-25 with the following replacement paragraph:

The present invention provides a yeast cell-surface display system successful in expressing the T cell receptor. Second, expression of the full length T cell receptor could only be achieved after randomly mutagenizing the T cell receptor gene and then selecting by flow cytometry for surface expression. This method thereby exploited an evolutionary approach to "correcting" the expression defect in the T cell receptor.

Please replace the last paragraph on page 81, line 26, through page 82, line 5 with the following replacement paragraph:

This same approach could be applied to any polypeptide which in its wild-type form is not displayed efficiently. Selection for "displayability" has been reduced to practice for the T cell receptor, as described in examples 33-37. Once displayable mutant versions of the polypeptide are obtained, these versions can then be subjected to the screening processes for improved binding properties that are described in examples 1-32.

Please replace the paragraph on page 82, lines 6-14 with the following replacement paragraph:

Improved T cell receptor molecules are useful in therapies for cancer, sepsis, and autoimmune diseases such as arthritis, diabetes, or multiple sclerosis. For example, soluble forms of high affinity T cell receptors would act as antagonists of detrimental T-cell mediated autoimmune diseases and thereby provide potential treatments for these diseases. Analogous strategies have been successfully employed with a soluble tumor necrosis factor receptor (TNF-R) and forms of this receptor are in clinical trials for septic shock and rheumatoid arthritis (Moosmayer et al., 1995).

Please replace the last paragraph on page 82, lines 15-26 with the following replacement paragraph:

In the methods of the present invention, yeast surface display allows single chain T cell receptors to be engineered to bind with high affinity to MHC-peptide complexes or superantigens. Such molecules would find a variety of medical uses. Examples include, but are not limited to: 1) interfering with inappropriate T cell attacks on healthy tissue in autoimmune diseases such as arthritis, diabetes, and multiple sclerosis; 2) interfering with septic shock due to bacterial superantigen that interact with T cells, leading to massive inflammatory reactions; and 3) destruction of tumor cells that bear T cell receptor ligands (e.g. specific tumor peptide/MHC complexes) by using high affinity T cell receptor together with anti-CD3 bispecific agents to redirect T cells to attack the cancerous cells.

Please replace the first paragraph on page 83, lines 1-15 with the following replacement paragraph:

Plasmids and strains. The single-chain TCR gene (V(8.2-linker-V(3.1) gene joined by a modified 205 linker (Cho et al., 1995) was subcloned by PCR into the vector pCT-Script (Stratagene, La Jolla, CA) following the manufacturer's protocol. A 6-His epitope tag was included at the carboxy-terminus of the scTCR for purification purposes. The ~800-bp NheI/XhoI fragment containing the scTCR was excised from pCR-Script and ligated into the yeast surface display vector pCT202 containing a nine-residue epitope tag (HA) and the AGA2 open reading frame downstream of the inducible GAL1 promoter. The resultant construct was transformed by the lithium acetate (LiAc) transformation method of Gietz and Schiestl (Gietz et al., 1995) into the *S. cerevisiae* strain BJ5465 ( $\alpha$ ura3-52 trp1 leu2 $\Delta$ 1 his3 $\Delta$ 200 pep4::HIS2 prb $\Delta$ 1.6 can1 GAL; Yeast Genetic Stock Center, Berkeley, CA) containing a chromosomally integrated AGA1 controlled by the GAL1 promoter (strain EBY100).

Please replace the paragraph on page 83, beginning with "Example 34", through page 84, line 20 with the following replacement paragraph:

#### **EXAMPLE 34**

##### Production of an scTCR random mutant library

Approximately 50 ng of pCT202/scTCR were transformed into *E. coli* XL1-Red cells (Stratagene, La Jolla, CA) according to the manufacturer's protocol. Following a 1 hour induction in SOC medium, the recovery was centrifuged at 2000 rpm for 5 min. and resuspended in 500  $\mu$ l of liquid LB medium containing 100  $\mu$ g/ml ampicillin plus 50  $\mu$ g/ml carbenicillin (LB-AMP100-CARB50). The resuspension was added to 15-ml LB-AMP100-CARB50 in a 50-ml Erlenmeyer flask and grown at 37°C with shaking. The culture was replenished with a fresh 15-ml LB-AMP100-CARB50 at mid-log phase ( $OD_{600}$  (0.2-0.4), then grown to saturation ( $OD_{600}$  ~1.0-1.1; this was considered one "cycle" or round of mutation). A small fraction of this culture (0.75  $\mu$ l) was added to the next cycle (15-ml LB-AMP100-CARB50). After six cycles of growth, Wizard Miniprep (Promega, Madison, WI) DNA plasmid preparations were performed on the 15-ml culture. Approximately 10  $\mu$ g of pCT202/scTCR DNA from cycle six were transformed into each of 10 tubes of yeast strain EBY100 using the LiAc method. The 10 reactions were pooled after resuspension in 1-ml ddH<sub>2</sub>O/tube, 1/10,000 of the pool plated on selective pates to determine transformation efficiency. The library size was approximately  $7 \times 10^6$ . A 50 ml volume of SD-CAA (glucose 2 wt %, Difco yeast nitrogen base 0.67 wt %, casamino acids 0.5 wt %) was inoculated with the remainder of the culture, grown overnight at 30°C with shaking, passaged to  $OD_{600}$  = 0.05, and grown overnight at 30°C to  $OD_{600}$  > 1.0. Five milliliters of selective galactose medium SG-CAA (where 2% galactose replaces the glucose in SD-CAA) were then inoculated to  $OD_{600}$  = 0.5 and grown overnight at 20°C with shaking for ~20-24 h (1-2 doublings).

Please replace the paragraph on page 84, beginning with "Example 35", through page 85, line 15 with the following replacement paragraph:

#### **EXAMPLE 35**

##### **Selection of scTCR mutant library by fluorescence-activated cell sorting**

Cells were labeled with 25  $\mu$ L Mab 1B2 (anti-V $\beta$ 8.2V $\alpha$ 3.1; prepared from ascites fluid and conjugated to biotin) at a concentration of 20  $\mu$ g/ml. Samples were sorted on a Coulter 753 bench with an event rate of ~4,000 cells/sec (Flow Cytometry Center, UIUC Biotechnology Center). A total of  $6 \times 10^7$  cells were examined during the first sorting round, with ~5% of the population collected. The collected cells were regrown between sorts at 30°C in 4 ml selective glucose medium SD-CAA. After ~18-20 hours, recombinant AGA1 + AGA2-scFv expression was induced at 20°C with shaking in 5 ml SG-CAA. A total of 3 rounds of sorting was performed, with the first sort in enrichment mode (high recovery of all positive clones) and the last 2 sorts in purification mode (coincident negative cells rejected). Immediately following the last sort, the collected cells were re-sorted, collected as two separate populations ("high expression" and "low expression"), and plated on selective plates to isolate individual clones. Twenty clones were examined by flow cytometry.

Please replace the paragraph on page 85, beginning with "Example 36", through page 86, line 13 with the following replacement paragraph:

#### **EXAMPLE 36**

##### **Induction and detection of mutant scTCR on the yeast surface**

Individual clones from the pCT202/scTCR library sorting were grown overnight at 30°C with shaking in 3 ml SD-CAA followed by induction in SG-CAA as described above. Cultures were harvested after (20-24 hours (1-2 doublings) by centrifugation, washed with PBS (10 mM NaPO<sub>4</sub>, 150 mM NaCl, pH 7.3) containing 0.1% bovine serum albumin and 0.05% azide, and incubated 45 minutes on ice with 25 (L of 10  $\mu$ g/ml anti-HA Mab 12CA5 (Boehringer Mannheim, Indianapolis, IN), or biotinylated-1B2 Mab (20  $\mu$ g/ml) prepared from ascites fluid. Cells were washed with PBS and incubated 30 minutes on ice with either FITC-labeled F(ab')<sub>2</sub> goat anti-mouse IgG (1:50; Kirkegaard and Perry Labs, Inc., Gaithersburg, MD) or a streptavidin-phycoerythrin (SA-PE) conjugate (1:100; PharMingen, San Diego, CA). Labeled yeast cells were analyzed on a Coulter Epics XL flow cytometer. Event rate was ~250 cells/sec. Data for 10,000 events was collected, and the population was gated according to light scatter (size) to prevent analysis of cell clumps. Results from the wild type (wt) TCR and several representative TCR mutants are shown in Figure 19. Double mutants containing the combined mutations from several of these isolates were also constructed and the results of flow cytometry of these are shown in Figure 20.

Please replace the paragraph on page 86, beginning with "Example 37", through page 87, line 6 with the following replacement paragraph:

### **EXAMPLE 37**

#### **Rescue and sequencing of mutant scTCR genes**

Plasmids from scTCR yeast (wt and 20 mutants) were rescued according to the protocol described by Ward (Ward, 1991), except that cells were disrupted with a bead beater (BioSpec Products, Inc., Bartlesville, OK) for 2 minutes instead of vortexing. Cells were centrifuged for 1 minute and the upper (aqueous) phase collected. A Wizard DNA Clean-Up kit (Promega, Madison, WI) was used to prepare the plasmid DNA and *E. coli* DH5 $\alpha$  ElectroMAX competent cells (GibcoBRL, Gaithersburg, MD) were transformed via electroporation with 1  $\mu$ l of the DNA preparation. Transformations were plated on LB-AMP50. Sequencing of wt scTCR and 20 mutants (mTCR1-mTCR20) was performed using primers that flank the scTCR of the display vector and fluorescence automated sequencing (Genetic Engineering Facility of the UIUC Biotechnology Center). Single mutations were found in the TCR for each of the isolates shown (Figure 21). These mutations may allow the expression of more stable TCR for possible therapeutic uses.

Please replace the paragraph on page 87, lines 8-27, with the following replacement paragraph:

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Please replace page 88, lines 1-27, with the following replacement paragraph:



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Please replace page 88, lines 1-27, with the following replacement paragraph:

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Please replace page 90, lines 1-27, with the following replacement paragraph:

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Please replace page 91, lines 1-18, with the following replacement paragraph:

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Please replace the paragraph on page 91, lines 19-23, with the following replacement paragraph:

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These references are incorporated by reference to the same extent as if each individual publication was specifically and individually incorporated by reference.

Please replace the last paragraph on page 91, line 24 through page 92, line 6, with the following replacement paragraph:

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples, along with the methods, procedures, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.